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<b>(54) Title:</b> IMMUNOMODULATORY PEPTIDES  <b>(57) Abstract</b>  A purified preparation of a peptide comprising an amino acid sequence identical to that of a segment of a naturally-occurring human protein, said segment being of 10 to 30 residues in length, inclusive, wherein said peptide binds to a human major histocompatibility complex (MHC) class II allotype.		

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IMMUNOMODULATORY PEPTIDES

The field of the invention is major histocompatibility complex (MHC) antigens.

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Background of the Invention

Major histocompatibility complex (MHC) class II antigens are cell surface receptors that orchestrate all specific immune responses in vertebrates. Humans possess three distinct MHC class II isotypes: DR, for which  
10 approximately 70 different allotypes are known; DQ, for which 33 different allotypes are known; and DP, for which 47 different allotypes are known. Each individual bears two to four DR alleles, two DQ alleles, and two DP alleles.

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MHC receptors (both class I and class II) participate in the obligate first step of immune recognition by binding small protein fragments (peptides) derived from pathogens or other non-host sources, and presenting these peptides to the regulatory cells (T  
20 cells) of the immune system. In the absence of MHC presentation, T cells are incapable of recognizing pathogenic material. Cells that express MHC class II receptors are termed antigen presenting cells (APC). APCs ingest pathogenic organisms and other foreign  
25 materials by enveloping them in endosomic vesicles, then subjecting them to enzymatic and chemical degradation. Foreign proteins which are ingested by APCs are partially degraded or "processed" to yield a mixture of peptides, some of which are bound by MHC class II molecules that  
30 are en route to the surface. Once on the cell surface, MHC-bound peptides are available for T cell recognition.

MHC class II antigens are expressed on the surface of APCs as a trimolecular complex composed of an  $\alpha$  chain, a  $\beta$  chain, and a processed peptide. Like most  
35 polypeptides that are expressed on the cell surface, both

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$\alpha$  and  $\beta$  chains contain short signal sequences at their  $\text{NH}_2$  termini which target them to the endoplasmic reticulum (ER). Within the ER the class II  $\alpha/\beta$  chain complex associates with an additional protein termed the invariant chain (Ii). Association with Ii is proposed to block the premature acquisition of peptides (by blocking the peptide binding cleft of the MHC heterodimer), promote stable  $\alpha/\beta$  interaction, and direct subsequent intracellular trafficking of the complex to endosomal vesicles. In the endosomes, Ii is removed by a process involving proteolysis; this exposes the peptide binding cleft, thus allowing peptides present in the endosome to bind to the MHC molecule. The class II/ peptide complex is transported from the endosomes to the cell surface where it becomes accessible to T-cell recognition and subsequent activation of immune responses. Class II MHC molecules bind not only to peptides derived from exogenous (ingested) proteins, but also to those produced by degradation of endogenous (self) proteins. The amount of each species of peptide which binds class II is determined by its local concentration and its relative binding affinity for the given class II binding groove, with the various allotypes displaying different peptide-binding specificities.

Early during fetal development, the mammalian immune system is "tolerized", or taught not to react, to self-peptides. The stability and maintenance of this system is critical for ensuring that an animal does not generate an immune response against self. A breakdown of this system gives rise to autoimmune conditions such as diabetes, rheumatoid arthritis and multiple sclerosis. Current technologies intended to manipulate the immune system into reestablishing proper nonresponsiveness include protocols involving the intravenous delivery of synthetic, high affinity binding peptides as blocking peptides.

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Vaccination can generate protective immunity against a pathogenic organism by stimulating an antibody-mediated and/or a T cell-mediated response. Most of the current vaccination strategies still use relatively crude  
5 preparations, such as attenuated or inactivated viruses. These vaccines often generate both antibody- and cell-mediated immunity, and do not allow one to modulate the type of immune response generated. Moreover, in many diseases the generation of the wrong type of response can  
10 result in an exacerbated disease state.

#### Summary of the Invention

In the work disclosed herein, naturally processed peptides bound to six of the some 70 known human MHC class II DR allotypes (HLA-DR1, HLA-DR2, HLA-DR3, HLA-  
15 DR4, HLA-DR7, and HLA-DR8) have been characterized. These peptides were found to be predominantly derived from self proteins rather than foreign proteins. Several self peptide families have been identified with the unexpected property of degenerate binding: that is, a  
20 given self-peptide will bind to a number of HLA-DR allotypes. This observation runs counter to the widely-accepted view of MHC class II function, which dictates that each allotype binds a different set of peptides. Furthermore, many if not all of the self-peptides  
25 disclosed herein bind to the class II molecules with relatively high affinity. These three characteristics-- (1) self rather than foreign, (2) degeneracy, and (3) high affinity binding--suggest a novel means for therapeutic intervention in disease conditions  
30 characterized by autoreactivity, such as Type I diabetes, rheumatoid arthritis, and multiple sclerosis. In addition, such therapy could be used to reduce transplant rejection.

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In the therapeutic methods of the invention, short peptides modelled on the high-affinity immunomodulating self peptides of the invention (which preferably are nonallelically restricted) are introduced into the APCs of a patient. Tissue typing to determine the particular class II alleles expressed by the patient may be unnecessary, as the peptides of the invention are bound by multiple class II isotypes. It may be useful to employ a "cocktail" of peptides, where complete degeneracy is lacking for individual peptides, i.e., where peptides binds to fewer than all allotypes; the cocktail provides overlapping binding specificity. Once in the APC, a peptide binds to the class II molecules with high affinity, thereby blocking the binding of immunogenic peptides which are responsible for the immune reaction characteristic of the disease condition. Because the blocking peptides of the invention are self peptides with the exact carboxy and amino termini tolerized during ontogeny, they are immunologically inert and will not induce an immune response which may complicate treatment using non-self blocking peptides.

The peptides of the invention may be introduced into APCs directly, e.g., by intravenous injection of a solution containing one or more of the peptides. Alternatively, the APCs may be provided with a means of synthesizing large quantities of the blocking peptides intracellularly. Recombinant genes that encode ER and/or endosomal targeting signals fused to blocking peptide sequences are linked to appropriate expression control sequences and introduced into APCs. Once in the cell, these genes direct the expression of the hybrid peptides. Peptides targeted to the ER will bind class II  $\alpha$  and  $\beta$  chains as they are translated and assembled into heterodimers. The presence of high affinity binding peptides within the ER will prevent association of the

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$\alpha/\beta$  complex with invariant chain, and thus interfere with intracellular trafficking. The class II molecule/blocking peptide complex may subsequently be expressed on the cell surface, but would not elicit an immune response since T cells are tolerized to this complex early in development. The use of peptides tagged with ER retention signals may also prevent the peptide-complexed class II molecules from leaving the ER. Alternatively, the recombinant peptide may be tagged with an endosomal targeting signal which directs it to the endosomal compartment after synthesis, thereby also skewing the ratio of endogenously-processed peptide to blocking peptide in the endosome and favoring binding of the high affinity blocking peptide to any class II molecules which did not bind it in the ER. It may be advantageous, for any individual patient, to employ one or more ER-directed peptides in combination with one or more endosome-directed peptide, so that  $\alpha$ - $\beta$  complexes which are not filled in the ER with peptides of the invention are then blocked in the endocytic pathway. The end result again is cell surface expression of a non-immunogenic class II/peptide complex.

The use of a class II nonrestricted high affinity binding peptide coupled to an intracellular delivery system permits the specific down-regulation of class II restricted immune responses without invoking the pleiotropic adverse reactions associated with the current pharmacological strategies. Successful application of these technologies will constitute a significant advance towards the treatment of autoimmune disease and prevention of transplant rejection.

The intracellular delivery system of the invention can also be utilized in a novel method of vaccination of an animal, e.g., a human patient or a commercially significant mammal such as a cow which is susceptible to

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diseases such as hoof and mouth disease. Such a system can be tailored to generate the type of immune response required in a given situation by adjustments in the following: (a) peptide specificity for class I or class 5 II MHC; (b) peptide/protein length and/or sequence, and (c) using specific tags for organelle targeting. The system of the invention ensures that peptides are produced only within cells, and are not present outside the cells where they could stimulate antibody production 10 by contact with B cells. This limits the immune response generated by such a vaccine to T cell-mediated immunity, thereby preventing either an inappropriate or potentially deleterious response as might be observed with standard vaccines targeting the organisms which cause, for 15 example, HIV, malaria, leprosy, and leishmaniasis. Furthermore, this exclusively T cell-mediated immune response can be class I or class II-based, or both, depending upon the length and character of the immunogenic peptides: MHC class I molecules are known to 20 bind preferentially to peptides 8 to 10 residues in length, while class II molecules bind with high affinity to peptides that range from 12 to 25 residues long.

Immunization and therapy according to the invention can employ a purified preparation of a peptide 25 of the invention, i.e., a peptide which includes an amino acid sequence identical to that of a segment of a naturally-occurring human protein (i.e., a "self protein"), such segment being of 10 to 30 residues in length, wherein the peptide binds to a human MHC class II 30 allotype, and preferably binds to at least two distinct MHC class II allotypes (e.g., any of the approximately 70 known DR allotypes, approximately 47 known DP allotypes, or approximately 33 known DQ allotypes). The portion of the peptide corresponding to the self protein segment is 35 herein termed a "self peptide". By "purified



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preparation" is meant a preparation at least 50% (by weight) of the polypeptide constituents of which consists of the peptide of the invention. In preferred embodiments, the peptide of the invention constitutes at least 60% (more preferably at least 80%) of the purified preparation. The naturally-occurring human protein is preferably HLA-A2 (as broadly defined below), HLA-A29, HLA-Bw62, HLA-C, HLA-DR $\alpha$ , HLA-DR $\beta$ , invariant chain (Ii), Ig kappa chain C region, Ig heavy chain, Na<sup>+</sup>/K<sup>+</sup> ATPase, transferrin, transferrin receptor, calcitonin receptor, carboxypeptidase E, MET kinase-related transforming protein, guanylate-binding protein, mannose-binding protein, apolipoprotein B-100, cathepsin C, cathepsin S, metalloproteinase inhibitor 1 precursor, or heat shock cognate 71 kD protein; it may be an MHC class I or II antigen protein or any other human protein which occurs at the cell surface of APCs. The self peptide preferably conforms to the following motif: at a first reference position (I) at or within 12-residues of the amino terminal residue of the segment, a positively charged residue (i.e., Lys, Arg, or His) or a large hydrophobic residue (i.e., Phe, Trp, Leu, Ile, Met, Tyr, or Pro; and at position I+5, a hydrogen bond donor residue (i.e., Tyr, Asn, Gln, Cys, Asp, Glu, Arg, Ser, Trp, or Thr). In addition, the peptide may also be characterized as having, at positions I+9, I+1, and/or I-1, a hydrophobic residue (i.e., Phe, Trp, Leu, Ile, Met, Pro, Ala, Val, or Tyr) (+ denotes positions to the right, or toward the carboxy terminus, and - denotes positions to the left, or toward the amino terminus.) A typical peptide of the invention will include a sequence corresponding to residues 31-40 (i.e., TQFVRFDSDA) or residues 106-115 (i.e., DWRFLRGYHQ) of HLA-A2, or residues 107-116 (i.e., RMTPLLMQA) of Ii, or

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a sequence essentially identical to any one of the sequences set forth in Tables 1-10 below.

The therapeutic and immunization methods of the invention can also employ a nucleic acid molecule (RNA or  
5 DNA) encoding a peptide of the invention, but encoding less than all of the entire sequence of the self protein. The nucleic acid preferably encodes no substantial portion of the self protein other than the specified self peptide which binds to a MHC class II molecule, although  
10 it may optionally include a signal peptide or other trafficking sequence which was derived from the self protein (or from another protein). A trafficking sequence is an amino acid sequence which functions to control intracellular trafficking (directed movement from  
15 organelle to organelle or to the cell surface) of a polypeptide to which it is attached. Such trafficking sequences might traffic the polypeptide to ER, a lysosome, or an endosome, and include signal peptides (the amino terminal sequences which direct proteins into  
20 the ER during translation), ER retention peptides such as KDEL; and lysosome-targeting peptides such as KFERQ, QREFK, and other pentapeptides having Q flanked on one side by four residues selected from K, R, D, E, F, I, V, and L. An example of a signal peptide that is useful in  
25 the invention is a signal peptide substantially identical to that of an MHC subunit such as class II  $\alpha$  or  $\beta$ ; e.g., the signal peptide of MHC class II  $\alpha$  is contained in the sequence MAISGVPVLGFFIIAVLMSAQESWA. The signal peptide encoded by the nucleic acid of the invention may include  
30 only a portion (e.g., at least ten amino acid residues) of the specified 25 residue sequence, provided that portion is sufficient to cause trafficking of the polypeptide to the ER. In preferred embodiments, the nucleic acid of the invention encodes a second self  
35 peptide and a second trafficking sequence (which may be

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identical to or different than the first self peptide and first trafficking sequence), and it may encode additional self peptides and trafficking sequences as well. In still another variation on this aspect of the invention, 5 the self peptide sequence (or a plurality of self peptide sequences arranged in tandem) is linked by a peptide bond to a substantially intact Ii polypeptide, which then carries the self peptide sequence along as it traffics the class II molecule from ER to endosome.

10 The nucleic acid of the invention may also contain expression control sequences (defined as transcription and translation start signals, promoters, and enhancers which permit and/or optimize expression of the coding sequence with which they are associated) and/or genomic 15 nucleic acid of a phage or a virus, such as an attenuated or non-replicative, non-virulent form of vaccinia virus, adenovirus, Epstein-Barr virus, or a retrovirus.

The peptides and nucleic acids of the invention may be prepared for therapeutic use by suspending them 20 directly in a pharmaceutically acceptable carrier, or by encapsulating them in liposomes, immune-stimulating complexes (ISCOMS), or the like. Such preparations are useful for inhibiting an immune response in a human patient, by contacting a plurality of the patient's APCs 25 with the therapeutic preparation and thereby introducing the peptide or nucleic acid into the APCs.

Also within the invention is a cell (e.g., a tissue culture cell or a cell, such as a B cell or APC, within a human) containing the nucleic acid molecule of 30 the invention. A cultured cell containing the nucleic acid of the invention may be used to manufacture the peptide of the invention, in a method which involves culturing the cell under conditions permitting expression of the peptide from the nucleic acid molecule.

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Disclosed herein is a method of identifying a nonallelically restricted immunomodulating peptide, which method includes the steps of:

- (a) fractionating a mixture of peptides eluted  
5 from a first MHC class II allotype;
- (b) identifying a self peptide from this mixture;  
and
- (c) testing whether the self peptide binds to a second MHC class II allotype, such binding being an  
10 indication that the self peptide is a nonallelically restricted immunomodulating peptide.

In further embodiments, the invention includes a method of identifying a potential immunomodulating peptide, in a method including the steps of:

- 15 (a) providing a cell expressing MHC class II molecules on its surface;
- (b) introducing into the cell a nucleic acid encoding a candidate peptide; and
- (c) determining whether the proportion of  
20 class II molecules which are bound to the candidate peptide is increased in the presence of the nucleic acid compared to the proportion bound in the absence of the nucleic acid, such an increase being an indication that the candidate peptide is a potential immunomodulating  
25 peptide.

Also within the invention is a method of identifying a potential immunomodulating peptide, which method includes the steps of:

- 30 (a) providing a cell expressing MHC class II molecules on its surface;
- (b) introducing into the cell a nucleic acid encoding a candidate peptide; and
- (c) determining whether the level of MHC class II molecules on the surface of the cell is decreased in the  
35 presence of the nucleic acid compared to the level of MHC

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class II molecules in the absence of the nucleic acid, such a decrease being an indication that the candidate peptide is a potential immunomodulating peptide.

Also included in the invention is a method of  
5 identifying a nonallelically restricted immunostimulating peptide, which method includes the steps of:

(a) providing a cell bearing a first MHC class I or class II allotype, such cell being infected with a pathogen (e.g., an infective agent which causes human or  
10 animal disease, such as human immunodeficiency virus (HIV), hepatitis B virus, measles virus, rubella virus, influenza virus, rabies virus, *Corynebacterium diphtheriae*, *Bordetella pertussis*, *Plasmodium spp.*, *Schistosoma spp.*, *Leishmania spp.*, *Trypanasoma spp.*, or  
15 *Mycobacterium lepre*);

(b) eluting a mixture of peptides bound to the cell's first MHC allotype;

(c) identifying a candidate peptide from the mixture, such candidate peptide being a fragment of a  
20 protein from the pathogen; and

(d) testing whether the candidate peptide binds to a second MHC allotype, such binding being an indication that the candidate peptide is a nonallelically restricted immunostimulating peptide. A nucleic acid  
25 encoding such an immunogenic fragment of a protein of a pathogen can be used in a method of inducing an immune response in a human patient, which method involves introducing the nucleic acid into an APC of the patient.

The therapeutic methods of the invention solve  
30 certain problems associated with prior art methods involving intravenous injection of synthetic peptides:  
(1) because of allelic specificity, a peptide capable of binding with high affinity to all, or even most, of the different class II allotypes expressed within the general

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population had not previously been identified; (2) the half-lives of peptides delivered intravenously are generally very low, necessitating repeated administration with the associated high level of inconvenience and cost; 5 (3) this type of delivery approach requires that the blocking peptide displace the naturally-occurring peptide occupying the binding cleft of a class II molecule while the latter is on the cell surface, which is now believed to be a very inefficient process; and (4) if the blocking 10 peptide utilized is itself immunogenic, it may promote deleterious immune responses in some patients.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

15

#### Detailed Description

The drawings are first briefly described.

#### Drawings

Figs. 1a-f are chromatographic analyses of the peptide pools extracted from papain digested HLA-DR1, 20 DR2, DR3, DR4, DR7, and DR8, respectively, illustrating the peptide repertoire of each HLA-DR as detected by UV absorbance. The UV absorbance for both 210 nm and 277 nm is shown at a full scale absorbance of 500 mAU with a retention window between 16 minutes and 90 minutes (each 25 mark represents 2 minutes).

Fig. 2 is a representative mass spectrometric analysis of the size distribution of isolated HLA-DR1 bound peptides. The determined peptide masses in groups of 100 mass units were plotted against the number of 30 isolated peptides identified by mass spectrometry. Peptide length was calculated by dividing the experimental mass by an average amino acid mass of 118 daltons.

Fig. 3 is a representation of two minigenes of the 35 invention, in which the HLA-DR $\alpha$  chain leader peptide is

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linked to the amino terminus of a 15-residue (A) or 24-residue (B) blocking peptide fragment of human invariant chain Ii.

### Experimental Data

#### 5 METHODS

##### I. Purification of HLA-DR antigens.

HLA-DR molecules were purified from homozygous, Epstein-Barr virus-transformed, human B lymphoblastoid lines: DR1 from LG-2 cells, DR2 from MST cells, DR3 from  
10 WT20 cells, DR4 from Priess cells, DR7 from Mann cells, and DR8 from 23.1 cells. All of these cell lines are publicly available. Cell growth, harvest conditions and protein purification were as previously described (Gorga, J. et al., 1991). Briefly, 200 grams of each cell type  
15 was resuspended in 10mM Tris-HCl, 1mM dithiothreitol (DTT), 0.1mM phenylmethylsulfonylfluoride (PMSF), pH 8.0, and lysed in a Thomas homogenizer. The nuclei were removed by centrifugation at 4000xg for 5 min and the pellets washed and repelleted until the supernatants were  
20 clear. All the supernatants were pooled and the membrane fraction harvested by centrifugation at 175,000xg for 40 min. The pellets were then resuspended in 10 mM Tris-HCl, 1mM DTT, 1mM PMSF, 4% NP-40. The unsolubilized membrane material was removed by centrifugation at  
25 175,000xg for 2 hours, and the NP-40 soluble supernatant fraction used in immunoaffinity purification.

Detergent soluble HLA-DR was bound to a LB3.1-protein A sepharose column (Gorga et al., *id*) and eluted with 100 mM glycine, pH 11.5. Following elution, the  
30 sample was immediately neutralized by the addition of Tris-HCl and then dialyzed against 10mM Tris-HCl, 0.1% deoxycholic acid (DOC). The LB3.1 monoclonal antibody recognizes a conformational determinant present on the

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nonpolymorphic HLA-DR $\alpha$  chain, and thus recognizes all allotypes of HLA-DR.

The transmembrane domain of the DR molecules was removed by papain digestion, and the resulting water-soluble molecule further purified by gel filtration chromatography on an S-200 column equilibrated in 10mM Tris-HCl, pH 8.0. The purified DR samples were concentrated by ultrafiltration, yield determined by BCA assay, and analyzed by SDS polyacrylamide gel electrophoresis.

## II. Extraction and fractionation of bound peptides.

Water-soluble, immunoaffinity-purified class II molecules were further purified by high-performance size exclusion chromatography (SEC), in 25 mM N-morpholino ethane sulfonic acid (MES) pH 6.5 and a flowrate of 1 ml/min., to remove any residual small molecular weight contaminants. Next, Centricon microconcentrators (molecular weight cutoff 10,000 daltons) (Amicon Corp.) were sequentially washed using SEC buffer and 10% acetic acid prior to spin-concentration of the protein sample (final volume between 100-200  $\mu$ l). Peptide pools were extracted from chosen class II alleles by the addition of 1 ml of 10% acetic acid for 15 minutes at 70°C. These conditions are sufficient to free bound peptide from class II molecules, yet mild enough to avoid peptide degradation. The peptide pool was separated from the class II molecule after centrifugation through the Centricon concentrator, with the flow-through containing the previously bound peptides.

The collected acid-extracted peptide pool was concentrated in a Savant Speed-Vac to a volume of 50  $\mu$ l prior to HPLC separation. Peptides were separated on a microbore C-18 reversed-phase chromatography (RPC) column (Vydac) utilizing the following non-linear gradient protocol at a constant flowrate of 0.15 ml/min.: 0-63



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min. 5%-33% buffer B; 63-95 min. 33%-60% buffer B; 95-105 min 60%-80% buffer B, where buffer A was 0.06% trifluoroacetic acid/water and buffer B was 0.055% trifluoroacetic acid/acetonitrile. Chromatographic analysis was monitored at multiple UV wavelengths (210, 254, 277, and 292 nm) simultaneously, permitting spectrophotometric evaluation prior to mass and sequence analyses. Shown in Fig.1 are chromatograms for each of the six DR peptide pools analyzed. Collected fractions were subsequently analyzed by mass spectrometry and Edman sequencing.

### III. Analysis of peptides.

The spectrophotometric evaluation of the peptides during RPC provides valuable information regarding amino acid composition (contribution of aromatic amino acids) and is used as a screening method for subsequent characterization. Appropriate fractions collected during the RPC separation were next analyzed using a Finnegan-MAT LaserMat matrix-assisted laser-desorption mass spectrometer (MALD-MS) to determine the individual mass values for the predominant peptides. Between 1%-4% of the collected fraction was mixed with matrix (1 $\mu$ l  $\alpha$ -Cyano-4-hydroxycinnamic acid) to achieve mass determination of extracted peptides. The result of this analysis for HLA-DR1 is shown in Fig. 2. Next, chosen peptide samples were sequenced by automated Edman degradation microsequencing using an ABI 477A protein sequencer (Applied Biosystems) with carboxy-terminal verification provided by mass spectral analysis using the Finnigan-MAT TSQ 700 triple quadrupole mass spectrometer equipped with an electro-spray ion source. This parallel analysis ensures complete identity of peptide composition and sequence. Peptide alignment with protein sequences stored in the SWISS-PROT database was performed using the

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FASTA computer database search program. Set forth in Tables 1-10 are the results of this sequence analysis for each of the DR molecules studied.

## RESULTS

### 5 I. HLA-DR1.

The HLA-DR1 used in this study was papain solubilized to enable the material to be used both for crystallographic and bound peptide analyses. The peptides bound to DR1 were acid extracted and  
10 fractionated using RPC (Fig. 1). The absence of any detectable peptidic material following a second extraction/RPC separation verified quantitative peptide extraction. Amino acid analysis (ABI 420A/130A derivatizer/HPLC) of extracted peptide pools demonstrated  
15 a 70-80% yield, assuming total occupancy of purified DR1 with a molar equivalent of bound peptides corresponding to the size distribution determined by mass spectrometry (see Fig. 2). The RPC profiles obtained from DR1 extractions of multiple independent preparations were  
20 reproducible. Furthermore, profiles from either detergent-soluble or papain-solubilized DR1 were equivalent. To confirm that the peptides were in fact identical in detergent-soluble and papain-digested DR1, mass spectrometry and Edman sequencing analyses were  
25 performed and revealed identical masses and sequences for analogous fractions from the two preparations.

Matrix-assisted laser desorption mass spectrometry (MALD-MS) was used to identify 111 species of unique mass contained within the eluted peptide pool of DR1 with an  
30 average size of 18 and a mode of 15 residues (Fig. 2). Over 500 additional mass species present within the molecular weight range of 13-25 residues were detected; however, the signal was not sufficient to assign individual masses with confidence. Multiple species of

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varying mass were detected in fractions corresponding to single RPC peaks indicating co-elution of peptides. To characterize these peptides further, samples were analyzed in parallel on a triple quadrupole mass spectrometer equipped with an electrospray ion source (ESI-MS) and by automated Edman degradation microsequencing (Lane et al., J. Prot. Chem. 10:151-160 (1991)). Combining these two techniques permits crucial verification of both the N- and C-terminal amino acids of peptides contained in single fractions. The sequence and mass data acquired for twenty peptides isolated from DR1 are listed in Table 1. All the identified peptides aligned with complete identity to regions of proteins stored in the SWISS-PROT database.

Surprisingly, sixteen of the twenty sequenced DR1-bound peptides were 100% identical to regions of the self proteins HLA-A2 and class II-associated invariant chain (Ii), representing at least 26% of the total extracted peptide mass. These isolated peptides varied in length and were truncated at both the N- and C-termini, suggesting that: 1) antigen processing occurs from both ends after binding to DR1, or 2) class II molecules bind antigen from a pool of randomly generated peptides. The yields from the peptide microsequencing indicated that HLA-A2 (Fig. 1) and Ii each represents at least 13% of the total DR1-bound peptides.

An additional surprising finding concerned a peptide which, although bound to HLA-DR and 100% homologous with HLA-A2 peptide, was derived from a cell which does not express HLA-A2 protein. Evidently this peptide is derived from a protein containing a region homologous with a region of HLA-A2 protein. Thus, for purposes of this specification, the term "HLA-A2 protein" is intended to include HLA-A2 protein itself, as well as any naturally occurring protein which contains a ten or

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greater amino acid long region of >80% homology with an HLA-DR-binding peptide derived from HLA-A2. An "HLA-A2 peptide" similarly refers to peptides from any HLA-A2 protein, as broadly defined herein.

5           The other four peptides identified in the DR1 studies were derived from two self proteins, transferrin receptor and the Na<sup>+</sup>/K<sup>+</sup> ATPase, and one exogenous protein, bovine serum fetuin (a protein present in the serum used to fortify the medium which bathes the cells). Each of  
10 these peptides occupied only 0.3-0.6% of the total DR1 population, significantly less than either the HLA-A2 or the Ii peptides. It is known that class II molecules en route to the cell surface intersect the pathway of incoming endocytic vesicles. Both recycling membrane  
15 proteins and endocytosed exogenous protein travel this common pathway. Hence, the HLA-A2, transferrin receptor, Na<sup>+</sup>/K<sup>+</sup> ATPase and bovine fetuin derived peptides would all encounter DR1 in a similar manner. Ii associates with nascent class II molecules in the endoplasmic reticulum  
20 (ER) (Jones et al., Mol. Immunol. 16:51-60 (1978)), preventing antigen binding until the class II/Ii complex arrives at an endocytic compartment (Roche and Cresswell, Nature 345:615-618 (1990)), where Ii undergoes proteolysis (Thomas et al., J. Immunol. 140:2670-2675  
25 (1988); Roche and Cresswell, Proc. Natl. Acad. Sci. USA 88:3150-3154 (1991)), thus allowing peptide binding to proceed. Presumably, the Ii peptides bound to DR1 were generated at this step.

          Synthetic peptides corresponding to five of the  
30 peptides reported in Table 1 were made and their relative binding affinities to DR1 determined. The influenza A hemagglutinin peptide (HA) 307-319 has been previously described as a high affinity, HLA-DR1 restricted peptide (Roche and Cresswell, J. Immunol. 144:1849-1856 (1990);  
35 Rothbard et al., Cell 52:515-523 (1988)), and was thus

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chosen as the control peptide. "Empty" DR1 purified from insect cells expressing recombinant DR1 cDNA was used in the binding experiments because of its higher binding capacity and 10-fold faster association kinetics than DR1 isolated from human cells (Stern and Wiley, Cell 68:465-477 (1992)). All the synthetic peptides were found to compete well ( $K_i < 100$  nM) against the HA peptide (Table 2). At first approximation, the Ii 106-119 peptide had the highest affinity of all the competitor peptides measured, equivalent to that determined for the control HA peptide. In addition to the  $K_i$  determinations, these peptides were found to confer resistance to SDS-induced  $\alpha$ - $\beta$  chain dissociation of "empty" DR1 when analyzed by SDS-PAGE, indicative of stable peptide binding (Sadegh-Nasseri and Germain, Nature 353:167-170 (1991); Dornmair et al., Cold Spring Harbor Symp. Quant. Biol. 54:409-415 (1989); Springer et al., J. Biol. Chem. 252:6201-6207 (1977)). Neither of the two control peptides,  $\beta_2m$  52-64 nor Ii 96-110, was able to either confer resistance to SDS-induced chain dissociation of DR1 or compete with HA 307-319 for binding to DR1; both of these peptides lack the putative binding motif reported in this study (see below).

A putative DR1 binding motif based on the sequence alignments of the core epitopes (the minimum length) of certain naturally processed peptides is shown in Table 3. The peptides listed in this table include those determined herein for HLA-DR1, as well as a number of peptides identified by others and known to bind DR1 (reference #6 in this table being O'Sullivan et al., J. Immunol. 145:1799-1808, 1990; reference #17, Roche & Cresswell, J. Immunol. 144:1849-1856, 1990; reference #25, Guttinger et al., Intern. Immunol. 3:899-906, 1991; reference #27, Guttinger et al. EMBO J. 7:2555-2558, 1988; and reference #28, Harris et al., J. Immunol.

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- 148:2169-2174, 1992). The key residues proposed in the motif are as follows: a positively charged group is located at the first position, referred to here as the index position for orientation (I); a hydrogen bond donor  
5 is located at I+5; and a hydrophobic residue is at I+9. In addition, a hydrophobic residue is often found at I+1 and/or I-1. Every naturally processed peptide sequenced from DR1 conforms to this motif (with the exception of the HLA-A2 peptide 103-116 that lacks residue I+9).
- 10 Because the putative motif is not placed in a defined position with respect to the first amino acid and because of the irregular length of bound peptides, it is impossible to deduce a motif from sequencing of peptide pools, as was done for class I molecules (Falk et al.,  
15 Nature 351:290-296 (1991)). The Ii 96-110 peptide, a negative control peptide used in binding experiments, has the I and I+5 motif residues within its sequence, but is missing eight additional amino acids found in Ii 105-118 (Table 3C).
- 20 A sequence comparison of 35 previously described DR1-binding synthetic peptides (O'Sullivan et al., J. Immunol. 145:1799-1808 (1990); Guttinger et al., Intern. Immunol. 3:899-906 (1991); Hill et al., J. Immunol. 147:189-197 (1991); Guttinger et al., EMBO J. 7:2555-2558  
25 (1988); Harris et al., J. Immunol. 148:2169-2174 (1992)) also supports this motif. Of the 35 synthetic peptides, 21 (60%) have the precise motif, nine (30%) contain a single shift at either I or I+9, and the remaining five (10%) have a single substitution at I (Table 3B and C).
- 30 Interestingly, in the latter peptides, a positive charge at I is always replaced by a large hydrophobic residue (Table 8C); a pocket has been described in class I molecules that can accommodate this precise substitution (Latron et al., Proc. Natl. Acad. Sci. USA 88:11325-11329  
35 (1991)). Contributions by the other eight amino acids

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within the motif or the length of the peptide have not been fully evaluated and may compensate for shifted/missing residues in those peptides exhibiting binding. Evaluation of the remaining 117 non-DR1 binding peptides cited in those studies (which peptides are not included in Table 3) indicates that 99 (85%) of these peptides do not contain the DR1 motif proposed herein. Of the remaining 18 peptides (15%) that do not bind to DR1 but which do contain the motif, 6 (5%) are known to bind to other DR allotypes; the remaining 12 peptides may have unfavorable interactions at other positions which interfere with binding.

In contrast to the precise N-terminal cleavages observed in the previous study of six peptides bound to the mouse class II antigen termed I-A<sup>b</sup> and five bound to mouse I-E<sup>b</sup> (Rudensky et al., Nature 356:622-627 (1991)), the peptides bound to DR1 are heterogeneous at both the N- and C-termini. In contrast to peptides bound to class I molecules, which are predominantly nonamers (Van Bleek and Nathenson, Nature 348:213-216 (1990); Rotzschke et al., Nature 348:252-254 (1990); Jardetzky et al., Nature 353:326-329 (1991); Hunt et al., Science 255:1261-1263 (1992)), class II peptides are larger and display a high degree of heterogeneity both in length and the site of terminal truncation, implying that the mechanisms of processing for class I and class II peptides are substantially different. Furthermore, the present results suggest that class II processing is a stochastic event and that a DR allotype may bind peptides of different lengths from a complex random mixture. The heterogeneity observed may be solely due to protection of bound peptides from further degradation. Thus, class II molecules would play an active role in antigen processing (as previously proposed (Donermeyer and Allen, J. Immunol. 142:1063-1068 (1989)) by protecting the bound

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peptides from complete degradation. Alternatively, the predominance of 15mers bound to DR1 (as detected by both the MALD-MS and the yields of sequenced peptides) could be the result of trimming of bound peptides. In any event, the absence of detectable amounts of peptides shorter than 13 and longer than 25 residues suggests that there are length constraints intrinsic either to the mechanism of peptide binding or to antigen processing. The predominance of peptides bound to DR1 that are derived from endogenously synthesized proteins, and particularly MHC-related proteins, may result from the evolution of a mechanism for presentation of self peptides in connection with the generation of self tolerance.

## 15 II. Other HLA-DR molecules.

The sequences of naturally processed peptides eluted from each of DR2, DR3, DR4, DR7 and DR8 are shown in Tables 4-8, respectively. Table 9 gives sequences of DR1 from another cell line which does not have wild-type Ar, but which has bound A2-like peptides. Table 10 gives sequences of peptides eluted from DR4 and DR11 molecules expressed in cells from a human spleen. These data demonstrate the great prevalence of self peptides bound, compared to exogenous peptides. The data also show that the A2 and Ii peptides occur repeatedly.

## III. Peptide Delivery

### Genetic Constructions.

In order to prepare genetic constructs for in vivo administration of genes encoding immunomodulatory peptides of the invention, the following procedure is carried out.



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Overlapping synthetic oligonucleotides were used to generate the leader peptide/blocking peptide mini-genes illustrated in Fig. 3 by PCR amplification from human HLA-DR $\alpha$  and invariant chain cDNA templates. These mini-genes encode the Ii peptide fragments KMRMATPLLMQALPM (or Ii<sub>15</sub>) and LPKPPKPVSKMRMATPLLMQALPM (or Ii<sub>24</sub>). The resulting constructs were cloned into pGEM-2 (Promega Corp.) to form the plasmids pGEM-2- $\alpha$ -Ii<sub>15</sub> and pGEM-2- $\alpha$ -Ii<sub>24</sub>, with an upstream T7 promoter for use in the in vitro transcription/translation system described below.

For in vivo expression, each mini-gene was subsequently subcloned from the pGEM-2 derivatives into a transfection vector, pH $\beta$ actin-1-neo (Gunning et al., (1987) P.N.A.S. U.S.A. 84:4831), to form the plasmids pH $\beta$ actin- $\alpha$ -Ii<sub>15</sub> and pH $\beta$ actin- $\alpha$ -Ii<sub>24</sub>. The inserted mini-genes are thus expressed in vivo from the constitutive/strong human  $\beta$  actin promoter. In addition, the mini-genes were subcloned from the pGEM-2 derivatives into the vaccinia virus recombination vector pSC11 (S. Chakrabarti et al. (1985) Mol. Cell Biol. 5, 3403-3409) to form the plasmids pSC11- $\alpha$ -Ii<sub>15</sub> and pSC11- $\alpha$ -Ii<sub>24</sub>. Following recombination into the viral genome the inserted mini-genes are expressed from the strong vaccinia p<sub>7.5</sub> promoter.

*Intracellular trafficking signals added to peptides.*

Short amino acid sequences can act as signals to target proteins to specific intracellular compartments. For example, hydrophobic signal peptides are found at the amino terminus of proteins destined for the ER, while the sequence KFERQ (and other closely related sequences) is known to target intracellular polypeptides to lysosomes, while other sequences target polypeptides to endosomes. In addition, the peptide sequence KDEL has been shown to act as a retention signal for the ER. Each of these

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signal peptides, or a combination thereof, can be used to traffic the immunomodulating peptides of the invention as desired. For example, a construct encoding a given immunomodulating peptide linked to an ER-targeting signal peptide would direct the peptide to the ER, where it would bind to the class II molecule as it is assembled, preventing the binding of intact Ii which is essential for trafficking. Alternatively, a construct can be made in which an ER retention signal on the peptide would help prevent the class II molecule from ever leaving the ER. If instead a peptide of the invention is targeted to the endosomic compartment, this would ensure that large quantities of the peptide are present when invariant chain is replaced by processed peptides, thereby increasing the likelihood that the peptide incorporated into the class II complex is the high-affinity peptides of the invention rather than naturally-occurring, potentially immunogenic peptides. The likelihood of peptides of the invention being available incorporation into class II can be increased by linking the peptides to an intact Ii polypeptide sequence. Since Ii is known to traffic class II molecules to the endosomes, the hybrid Ii would carry one or more copies of the peptide of the invention along with the class II molecule; once in the endosome, the hybrid Ii would be degraded by normal endosomal processes to yield both multiple copies of the peptide of the invention or molecules similar to it, and an open class II binding cleft. DNAs encoding immunomodulatory peptides containing targeting signals will be generated by PCR or other standard genetic engineering or synthetic techniques, and the ability of these peptides to associate with DR molecules will be analyzed *in vitro* and *in vivo*, as described below.

It is proposed that the invariant chain prevents class II molecules from binding peptides in the ER and

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may contribute to heterodimer formation. Any mechanism that prevents this association would increase the effectiveness of class II blockade. Therefore, a peptide corresponding to the site on Ii which binds to the class II heterodimer, or corresponding to the site on either the  $\alpha$  or  $\beta$  subunit of the heterodimer which binds to Ii, could be used to prevent this association and thereby disrupt MHC class II function.

*In Vitro Assembly.*

10 Cell free extracts are used routinely for expressing eukaryotic proteins (Krieg, P. & Melton, D. (1984) Nucl. Acids Res. 12, 7057; Pelham, H. and Jackson, R. (1976) Eur. J. Biochem. 67, 247). Specific mRNAs are transcribed from DNA vectors containing viral RNA  
15 polymerase promoters (Melton, D. et al. (1984) Nucl. Acids Res. 12, 7035), and added to micrococcal nuclease-treated cell extracts. The addition of  $^{35}\text{S}$  methionine and amino acids initiates translation of the exogenous mRNA, resulting in labeled protein. Proteins may be  
20 subsequently analyzed by SDS-PAGE and detected by autoradiography. Processing events such as signal peptide cleavage and core glycosylation are initiated by the addition of microsomal vesicles during translation (Walter, P. and Blobel, G. (1983), Meth. Enzymol., 96,  
25 50), and these events are monitored by the altered mobility of the proteins in SDS-PAGE gels.

The ability of peptides containing a signal peptide sequence to be accurately processed and to compete with invariant chain for class II binding in the  
30 ER are assayed in the *in vitro* system described above. Specifically, DR1 $\alpha$  and  $\beta$  chain, and invariant chain peptide constructs described above are transcribed into mRNAs, which will be translated in the presence of mammalian microsomal membranes. Association of the DR

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heterodimer with Ii is determined by immunoprecipitation with antisera to DR and Ii. Addition of mRNA encoding the peptide of the invention to the translation reaction should result in a decreased level of

- 5 coimmunoprecipitated Ii, and the concomitant appearance of coimmunoprecipitated peptide, as determined by SDS-PAGE on TRIS-Tricine gels. These experiments will provide us with a rapid assay system for determining the potential usefulness of a given blocking peptide as a
- 10 competitor for Ii chain binding in the ER. Those peptides of the invention which prove to be capable of competing successfully with Ii in this cell-free assay can then be tested in intact cells, as described below.

#### *In Vivo Assembly.*

- 15 Human EBV-transformed B cell lines LG-2 and HOM-2 (homozygous for HLA-DR1) and the mouse B cell hybridoma LK35.2 are transfected with either 50 $\mu$ g of linearized pH $\beta$ actin- $\alpha$ -Ii<sub>15</sub> or pH $\beta$ actin- $\alpha$ -Ii<sub>24</sub> or (as a control) pH $\beta$ actin-1-neo by electroporation (150mV, 960 $\mu$ F, 0.2cm
- 20 cuvette gap). Following electroporation, the cells are cultured in G418-free medium until total recovery (approximately 4 days). Each population is then placed under G418 selection until neomycin-expressing resistant populations of transfectants are obtained (approximately
- 25 1-2 months). The resistant populations are subcloned by limiting dilution and the clonality of stable transfectants determined by PCR amplification of blocking peptide mRNA expression.

- Stable transfectants of LG-2 and HOM-2 carrying
- 30 blocking peptide mini-genes or negative control vectors are grown in large-scale culture conditions until 20 grams of pelleted cell mass is obtained. The HLA-DR expressed by each transfectant is purified, and the bound peptide repertoire (both from within the cell and from

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the cell surface) analyzed as described above.

Successful demonstration of a reduction in the total bound peptide diversity will be conclusive evidence of intracellular delivery of immuno-modulatory peptides.

5           A second cell-based assay utilizes stable transfectants of LK35.2 cells carrying blocking peptide mini-genes or negative control vectors; these cells are used as APCs in T cell proliferation assays. Each transfectant is cultured for 24 hours in the presence of  
10 different dilutions of hen egg lysozyme (HEL) and HEL-specific T cell hybridomas. The relative activation of the T cells present in each assay (as measured by lymphokine production) is determined using the publicly available lymphokine dependent cell line CTLL2 in a <sup>3</sup>H-thymidine incorporation assay (Vignali et al. (1992) J.E.M. 175:925-932). Successful demonstration of a reduction in the ability of blocking peptide expressing transfectants to present HEL to specific T cell  
15 hybridomas will be conclusive evidence of intracellular delivery of immuno-modulatory peptides. Cells of the human TK<sup>-</sup> cell line 143 (ATCC) are infected with vaccinia virus (strain WR, TK<sup>+</sup>) (ATCC), and two hours postinfection, pSC11- $\alpha$ -Ii<sub>15</sub> or pSC11- $\alpha$ -Ii<sub>24</sub> or pSC11 is introduced into the infected cells by calcium phosphate  
20 precipitation. TK<sup>-</sup> recombinants are selected for with bromodeoxyuridine at 25 $\mu$ g/ml. Recombinant plaques are screened by PCR for the presence of mini-gene DNA. Recombinant virus is cloned by three rounds of limiting dilution to generate pure clonal viral stocks.

30           In experiments analogous to the transfection experiments described above, recombinant vaccinia viruses encoding mini-genes or vector alone will be used to infect large-scale cultures of the human EBV transformed B cell lines LG-2 and HOM-2. Following infection, the  
35 HLA-DR is purified and the bound peptide repertoire

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analyzed as described above. A reduction of the complexity of the bound peptide population and a significant increase in the relative amount of Ii peptides bound are conclusive evidence that vaccinia can  
5 deliver blocking peptides to human APCs.

The same recombinant vaccinia viruses encoding mini-genes or vector will be used to infect mice experiencing experimentally-induced autoimmunity. A number of such models are known and are referred in  
10 Kronenberg, Cell 65:537-542 (1991).

*Liposomal Delivery of Synthetic Peptides or Mini-gene Constructs.*

Liposomes have been successfully used as drug carriers and more recently in safe and potent adjuvant  
15 strategies for malaria vaccination in humans (Fries et al. (1992), Proc. Natl. Acad. Sci. USA 89:358). Encapsulated liposomes have been shown to incorporate soluble proteins and deliver these antigens to cells for both in vitro and in vivo CD8<sup>+</sup> mediated CTL response  
20 (Reddy et al., J. Immunol. 148:1585-1589, 1992; and Collins et al., J. Immunol. 148:3336-3341, 1992). Thus, liposomes may be used as a vehicle for delivering synthetic peptides into APCs.

Harding et al. (Cell (1991) 64, 393-401) have  
25 demonstrated that the targeting of liposome-delivered antigen to either of two intracellular class II-loading compartments, early endosomes and/or lysosomes, can be accomplished by varying the membrane composition of the liposome: acid-sensitive liposomes were found to target  
30 their contents to early endosomes, while acid-resistant liposomes were found to deliver their contents to lysosomes. Thus, the peptides of the invention will be incorporated into acid-sensitive liposomes where delivery

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to endosomes is desired, and into acid-resistant liposomes for delivery to lysosomes.

Liposomes are prepared by standard detergent dialysis or dehydration-rehydration methods. For acid-sensitive liposomes, dioleoylphosphatidylethanolamine (DOPE) and palmitoylhomocystein (PHC) are utilized, while dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylserine (DOPS) are used for the preparation of acid-resistant liposomes.  $10^{-5}$  mol of total lipid (DOPC/DOPS or DOPE/PHC at 4:1 mol ratios) are dried, hydrated in 0.2 ml of HEPES buffered saline (HBS) (150 mM NaCl, 1 mM EGTA, 10mM HEPES pH 7.4) and sonicated. The lipid suspensions are solubilized by the addition of 0.1 ml of 1 M octylglucoside in HBS. The peptides to be entrapped are added to 0.2 ml of 0.6 mM peptide in 20% HBS. The mixture is then frozen, lyophilized overnight, and rehydrated. These liposomes will be treated with chymotrypsin to digest any surface-bound peptide. Liposome delivery to EBV-transformed cell lines (as described above) will be accomplished by 12-16 hour incubation at 37°C. HLA-DR will be purified from the liposome treated cells and bound peptide analyzed as above.

Alternatively, the liposomes are formulated with the DNA mini-gene constructs of the invention, and used to deliver the constructs into APCs either in vitro or in vivo.

Human immunization will be carried out under the protocol approved by both The Johns Hopkins University Joint Committee for Clinical Investigation and the Human Subject Research Review Board of the Office of the Surgeon General of the U.S. Army (Fries et al. (1992), P.N.A.S. U.S.P. 89:358-362), using dosages described therein, or other dosages described in the literature for liposome-based delivery of therapeutic agents.

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*Delivery via Immune-stimulating Complexes (ISCOMS).*

ISCOMS are negatively charged cage-like structures of 30-40nm in size formed spontaneously on mixing cholesterol and Quil A (saponin). Protective immunity  
5 has been generated in a variety of experimental models of infection, including toxoplasmosis and Epstein-Barr virus-induced tumors, using ISCOMS as the delivery vehicle for antigens (Mowat and Donachie) *Immunology Today* 12:383-385, 1991. Doses of antigen as low as 1µg  
10 encapsulated in ISCOMS have been found to produce class I mediated CTL responses, where either purified intact HIV-1-IIIB gp 160 envelope glycoprotein or influenza hemagglutinin is the antigen (Takahashi et al. , *Nature* 344:873-875, 1990). Peptides are delivered into tissue  
15 culture cells using ISCOMS in a manner and dosage similar to that described above for liposomes; the class II peptide binding of delivered peptides are then determined by extraction and characterization as described above. ISCOM-delivered peptides of the invention which are  
20 effectively utilized by cultured cells are then tested in animals or humans.

In addition to delivery of the therapeutic synthetic peptides, ISCOMS could be constituted to deliver the mini-gene constructs to APCs, and thus serve  
25 as an alternative to the above-outlined vaccinia strategy.

*Immunogenic Peptide Delivery (Vaccines).*

In addition to using the above-described intracellular delivery systems to deliver nonimmunogenic  
30 self peptides with the specific aim of down-modulating the immune system (thus alleviating autoimmune conditions), the delivery systems of the invention may alternatively be used as a novel means of vaccination, in order to stimulate a portion of the immune system of an



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animal. In the latter context, the delivery system is employed to deliver, into appropriate cells, DNA constructs which express immunogenic, pathogen-derived peptides intended to stimulate an immune response against  
5 a specific pathogen. Because the antigenic peptide is produced inside the target cell itself, the vaccine method of the invention ensures that there is no circulating free antigen available to stimulate antibody formation and thereby induce potentially deleterious or  
10 inappropriate immunological reactions. The immune response stimulated by vaccines of the invention is, because the vaccines are targeted solely to APC's, limited to the T cell mediated response, in contrast to standard vaccine protocols which result in a more  
15 generalized immune response. Although some of the peptide-presenting APC's will initially be lysed by host T cells, such lysis will be limited because, inter alia, the virus-based vaccine is non-replicative, i.e., each carrier virus can infect only one cell.

20 The model antigen that will be used to perfect and test the system of the invention is hen egg lysozyme (HEL). It is arguably the most well characterized protein for antigen presentation studies, to which there are numerous monoclonal antibodies and class I- and class  
25 II-restricted mouse T cell clones and hybridomas. The primary epitopes that will be studied are the peptide HEL 34-45, as both monoclonal antibodies and CD4+ T cell hybridomas are available, and peptide HEL 46-61, as both class I and class II-restricted T cell clones and  
30 hybridomas have been raised and are publicly available. These two sequences are thus proven immunogenic epitopes. Initially, four constructs encoding different polypeptides are analyzed: (a) whole, secreted HEL, (B) HEL 34-45, (c) HEL 46-61, and (d) HEL 34-61. The last  
35 three include a signal sequence known to be cleaved in

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these cells, e.g., IA<sup>k</sup> (MPRSRALILGVLALT<sup>T</sup>TMLSLCGG), which would result in targeting to the ER. All constructs are then subcloned into pH $\beta$ Apr-1 neo. The methodology for making these constructs is similar to that outlined

5 above. The constructs are introduced into appropriate APCs, e.g., LK35.2 cells, by means of a conventional eukaryotic transfection or one of the delivery vehicles discussed above (e.g., vaccinia, liposomes, or ISCOMS). LK35.2 cells, which possess the mouse MHC Class II

10 restriction molecules IA<sup>k</sup> and IE<sup>k</sup>, transfected with each of the constructs are tested for their ability to stimulate the appropriate class I and class II-restricted T cell hybridomas and clones using standard techniques. Whether class I stimulation is observed will depend on

15 whether peptide trimming can occur in the ER, in order to produce an 8-10-mer suitable for binding to class I molecules. If these constructs are ineffective for class I stimulation, they can be modified in order to produce a more effective peptide for class I binding. If these

20 constructs prove to be less effective for class II-restricted responses, they can be tagged with endosomal and/or lysosomal targeting sequences as discussed in Section V.

The effectiveness of targeting signals used to

25 direct immunogenic peptides to particular intracellular organelles would be monitored using electron microscopic analysis of immunogold stained sections of the various transfectants. Rabbit anti-peptide antisera would be produced and affinity purified for this application. In

30 addition, monoclonal antibody HF10, which recognizes HEL 34-45, will be used.

Once a construct is defined that can be effectively presented by transfectants *in vitro*, its effectiveness *in vivo* will be determined. This can be

35 tested by injection of the transfectants *i.p.* and/or *s.c.*

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into C3H/Balb/c F1 mice, or by injection of the construct incorporated into an appropriate delivery vehicle (e.g., liposome, ISCOMS, retrovirus, vaccinia). Optimal protocols and doses for such immunizing injections can be  
5 determined by one of ordinary skill in the art, given the disclosures provided herein. Efficiency of immunization can be tested by standard methods such as (a) proliferation of class II-restricted T cells in response to HEL pulsed APCs, (b) CTL response to <sup>51</sup>Cr-labeled  
10 targets, and (c) serum antibody titre as determined by ELISA.

Once the details of the vaccine delivery system of the invention are optimized, constructs encoding peptides with useful immunizing potential can be incorporated into  
15 the system. Such peptides can be identified by standard means now used to identify immunogenic epitopes on pathogen-derived proteins. For example, candidate peptides for immunization may be determined from antibody and T cell analysis of animals infected with a particular  
20 pathogen. In order to obtain a protective and effective anamnestic response, the peptides used for vaccination should ideally be those which are presented with the highest frequency and efficiency upon infection. This could best be determined by using the procedures outlined  
25 in the experimental section above to extract and characterize the peptides bound by MHC class II molecules from infected cells. Given allelic restriction of immunogenic peptides (in contrast to the observed degenerate binding of self peptides of invention), a  
30 mini-gene encoding several immunogenic peptides will probably be required to provide a vaccine useful for the entire population. Vaccine administration and dosage are as currently employed to smallpox vaccination.

What is claimed is:

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TABLE 1  
LG-2/HLA-DRI BINDING PEPTIDES

PROTEIN SOURCE	POSITION	SEQUENCE	LENGTH	FRACTION	MW	MASS SPEC	YIELD
HLA-A2	103-120	VGSDURFLRGTHQAYDG	18	DR1S-59	2190.4	2190.4	39.5
	103-117	VGSDURFLRGTHQYA	15	DR1S-58	1855.0	1854.4	907.5
	103-116	VGSDURFLRGTHQY	14	DR1S-58	1784.0	1783.6	53.3
	104-117	GSURFLRGTHQYA	14	DR1S-56	1755.3	1755.2	96.5
	105-117	SDURFLRGTHQYA	13	DR1S-56	1698.2	1698.8	48.8
Invariant Chain (11)	96-120	LPKPPKPVSKHRMATPLLMOALPHG	25	DR1S-88	2733.5	2734.5	40.5
	96-119	LPKPPKPVSKHRMATPLLMOALPM	24	DR1S-88	2676.4	2675.9	80.8
	97-120	PKPPKPVSKHRMATPLLMOALPHG	24	DR1S-86	2620.2	2619.7	91.5
	96-118	LPKPPKPVSKHRMATPLLMOALP	23	DR1S-86	2545.2	2544.5	112.2
	97-119	PKPPKPVSKHRMATPLLMOALPM	23	DR1S-87	2563.2	2562.3	145.0
	98-119	KPPKPVSKHRMATPLLMOALPM	22	DR1S-87	2466.1	2465.8	101.5
	97-118	PKPPKPVSKHRMATPLLMOALP	22	DR1S-84	2432.0	2431.7	72.5
	98-118	KPPKPVSKHRMATPLLMOALP	21	DR1S-84	2334.9	2334.2	31.6
	98-118	PKPPKPVSKHRMATPLLMOALP	20	DR1S-86	2206.7	2207.4	89.8
	105-119	KHRMATPLLMOALPM	15	DR1S-88	1732.2	1731.9	178.5
	105-118	KHRMATPLLMOALP	14	DR1S-86	1601.0	1600.2	162.0
	199-216	IPADLRISANGCKVDNS	18	DR1S-56	1886.6	1885.8	48.8
	680-696	RVEYHFLSPYVSPKESP	17	DR1S-58	2035.3	2036.8	30.3
Bovine Fetuin	56-74	YKHTLNQIDSVKVVPRRPT	19	DR1S-51	2237.6	2236.5	62.0
	56-73	YKHTLNQIDSVKVVPRRP	18	DR1S-50	2338.7	2338.5	32.5
HLA-DR $\beta$ -chain	43-61	DVGEYRAVTELCRPOAEYV	19	DR1S-51	2226.5	?	?
Carboxypeptidase E	101-115	EPGEPEFKYIGNMHG	15	DR1S-48	1704.9	1700.4*	EST-MS

SUBSTITUTE SHEET

TABLE 2  
PEPTIDE BINDING TO HLA-DR1

PEPTIDE <sup>a</sup>	LENGTH	KI vs HA 307-319 <sup>b</sup> nM	SDS-Resistance <sup>c</sup>
HLA-A2 103-117	15	49 ± 3	+
II 105-119	15	< 10	+
II 96-119	24	33 ± 5	+
Na <sup>+</sup> /K <sup>+</sup> ATPase 199-216	18	68 ± 9	+
Transf. Recept. 680-696	17	< 10	+
Bovine Fetuin 56-72	19	66 ± 18	+
HA 307-319	14	< 10	+
II 96-110	15	> 10 <sup>4</sup>	-
$\beta_2^m$ 52-64	13	> 10 <sup>4</sup>	-

<sup>a</sup> The first six entries correspond to peptides found associated with HLA-DR1 and the sequences are shown in Table 1. Two control peptides were also tested:  $\beta_2^m$  52-64, SDLSKQMSFYL, is from human  $\beta_2$ -microglobulin and II 96-110, LKPKPVSDRRAT is a truncated version of the longest invariant chain derived peptide isolated from HLA-DR1. Peptides were synthesized using solid-phase fmoc chemistry, deprotected and cleaved using standard methods, then purified by RPC. Purified peptides were analyzed by mass spectrometry and concentrations were determined by quantitative ninhydrin analysis.

<sup>b</sup> Inhibition constants (KI) were measured as the concentration of test peptide which inhibited 50% of the <sup>125</sup>I-labeled HA 307-319 binding to "empty" HLA-DR1 produced in Sf9 insect cells (20). HA 307-319 was labeled using Na<sup>125</sup>I and chloramine-T and isolated by gel filtration. Specific activity, determined by BCA assay (Pierce) and gamma counting, was 26,000 cpm/pmol. 10nM labeled peptide and 10 nM purified HLA-DR1 were mixed with 10 different concentrations (10 nM to 10  $\mu$ M) of synthetic cold competitor peptide in phosphate-buffered saline, pH 7.2, containing 1 mM EDTA, 1mM PMSF, 0.1 mM Iodoacetamide, and 3 mM NaH<sub>2</sub>PO<sub>4</sub>, and incubated at 37°C for 85 hours. Free and bound peptide were separated by native gel electrophoresis (33) and bound radioactivity was quantitated using a Fujix imaging plate analyzer (BAS 2000) after four hour exposures on the phosphor-imaging plates. Percent inhibition was calculated as the ratio of background-corrected radioactivity in the sample to background-corrected radioactivity in a parallel sample containing no competitor peptide. Under these conditions, KI measurements < 10 nM could not be accurately determined.

<sup>c</sup> The ability of the synthetic peptides to confer resistance to SDS-induced chain dissociation of HLA-DR1 produced in insect cells was determined as described (20). Briefly, 20  $\mu$ M HLA-DR1 was incubated with five-fold excess of synthetic peptide at 37°C for 85 hours, in phosphate-buffered saline (pH 7.2) with the protease inhibitor mixture described above. After incubation, the samples were analyzed by SDS-PAGE with and without boiling prior to loading. Peptides which prevented SDS-induced chain dissociation are indicated positive (+) and those that did not negative (-).

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TABLE 3 - PUTATIVE HLA-DRI PEPTIDE BINDING MOTIF

A	PROTEIN SOURCE	PEPTIDE SEQUENCE	LENGTH	POSITION	REFERENCE
	HLA-A2	SDVRFLRGYNQYA	13	105-117	This study
	Invariant Chain	KMMATPLLRQALP	14	105-118	
	Na <sup>+</sup> /K <sup>+</sup> ATPase	IPADLRITISANGCKYONS	18	199-216	
	Transferrin Receptor	RVEYHFLSPYSPKESP	17	680-696	
	Bovine Fetuin	YEHTLNQIDSYKVPKRP	18	56-73	
	B HEL	KVFGRCCLAAAKRNGLD	18	1-18	6
		RNGCKGTQVQAVIRGRL	18	112-129	6
	$\beta_2^m$	HPPHIEIQMLKNGKXI	16	31-46	6
	PLA <sub>2</sub>	NELGRFKHTDAGCRTN	16	19-34	6
		SKPKYQYQFDRKY	14	115-128	6
	WASE	ATSTKLNKEPATLKAIDG	20	1-20	6
		PATLKAIDGQIVKLYKGG	20	11-30	6
		DRVKLNYGQPMIFRLLVQ	20	21-40	6
		VAYVYKPHNTHECHLKSEA	20	111-130	6
	HIV p13	QRQEPIDKELYPLTSL	16	97-112	6
	HIV p17	GAGASVLGGELQKWE	16	1-16	6
	Influenza HA	RILYQHVQTVSVGTSTLNK	20	187-206	6
	Influenza HA	PKYVKQNTLKAT	13	307-319	17
	P. falcip. p190	LKGLVFCYKPLQNI	15	249-263	25
	P. falcip. CS	KNIEQYLKKIKNS	13	329-341	27
	Chicken OVA	DVFKELSVHNHNEIIE	16	15-30	6
	DRI $\beta$ chain	GDTRPRFLQRLKECHFFNG	20	1-20	28
		TERVRLLEECIYNQESYRFD	22	21-42	28
		DLLEGRBAADVICHRYNYGVGESFT	25	64-90	28
	p Cyt c	KAEKALTAIYKQATAK	17	88-104	6
	Myelin basic prot.	GRTOOEHPVYVFFKNIVTPRTPPP	24	75-98	6
	C Influenza Matrix	PLKAEIAQRLEDV	13	19-31	6
	HIV p17	RQILGQLOPGLQTSSE	16	57-72	6
	$\beta_2^m$	IQVTSRHPPEKPKPI	16	7-22	6
	PLA <sub>2</sub>	INTKCYKLENPVIGCG	16	85-100	6

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Table 3, continued

A	PROTEIN SOURCE	PEPTIDE SEQUENCE	LENGTH	POSITION	REFERENCE
	P. falcip. p190	YKLNFIYDFLLRAKL	14	211-224	25
		IDTLKKNEIKEI	13	338-350	25
	DR1 $\beta$ chain	DVGEYRAVTELGPRDAEYUN	20	43-62	28
	HIV p17	ERFAVIRPGLLETSEGC	16	41-56	6
	HEL	DNYRGYSLGHWCAAKFESNFTG	23	20-42	6
	NASE	EALVRGGLAKVATYVYKPNNT	20	101-120	6
	HIV p25	PIVONHLOGGVHQAIS	16	1-16	6
		SALSEGAIPODLNTHL	16	41-56	6
	$\beta_2^m$	SFYLANIEFTETETG	16	61-76	6
	PLA <sub>2</sub>	KHYFNLIINTKCYKLEH	16	79-94	6

**TABLE 4**  
**INST/HLA-DR2 BINDING PEPTIDES**

PROTEIN SOURCE	POSITION	SEQUENCE	LENGTH	FRACTION	K <sub>D</sub>	MASS SPEC
Pseudo HLA-A2	103-120	VGSDURFLRGYHOAYDG	18	DR2-3-57	2190.4	2189.0
	103-119	VGSDURFLRGYHOAYAD	17	DR2-3-57	2133.3	2131.8
	104-120	GSDURFLRGYHOAYDG	17	DR2-3-56	2034.3	2040.4
	103-117	VGSDURFLRGYHOYA	15	DR2-3-56	1855.0	1858.5
	103-116	VGSDURFLRGYHOY	14	DR2-3-56	1784.0	1786.3
	104-117	GSDURFLRGYHOYA	14	DR2-3-55	1755.3	1755.0*
	105-117	SDURFLRGYHOYA	13	DR2-3-56	1698.2	1702.6
	96-119	LPKPKPVSIQRNATPLLMOALPH	24	DR2-3-70	2676.4	2675.0*
	97-119	PKPKPVSIQRNATPLLMOALPH	23	DR2-3-70	2563.2	2562.0*
	98-119	KPKPKPVSIQRNATPLLMOALPH	22	DR2-3-70	2466.1	2465.0*
Invariant Chain (11)	97-118	PKPKPVSIQRNATPLLMOALP	22	DR2-3-66	2432.0	2437.0
	98-118	KPKPKPVSIQRNATPLLMOALP	21	DR2-3-66	2334.9	2340.0
	99-118	PPKPKPVSIQRNATPLLMOALP	20	DR2-3-70	2206.7	2207.0*
	105-123	KQRNATPLLMOALPHGALP	19	DR2-3-71	2070.5	2074.3
	105-119	KQRNATPLLMOALPH	15	DR2-3-70	1732.2	1732.0*
	59-81	ENHIFLGATNYIVLNEEDLQKV	23	DR2-3-65	2746.1	2746.6
(NET) Kinase-related Transforming protein Guanylate-bind. Mannose-bind. prot. HLA-DR2a $\beta$ -chain	434-450	QELKIKYQVPRKGIQA	17	DR2-3-71	2063.4	2074.3
	174-193	IQNLKKEAFLGITDEKTEG	20	DR2-3-70	2248.5	2248.0*
	1-127	GDTRPRFLQOKYECFFNGTERVRL	127	DR2-3-65	15055	15097
	1-126	GDTRPRFLQOKYECFFNGTERVRL	126	DR2-3-66	14941	15013
	1-127	GDTRPRFLQOKYECFFNGTERVRL	127	DR2-3-70		7
	1-126	GDTRPRFLQOKYECFFNGTERVRL	126	DR2-3-71		15009
	94-111	RVQPKVTVPSTQPLQH	18	DR2-3-39	2106.5	2114.
	94-108	RVQPKVTVPSTQPLQ	15	DR2-3-39	1728.3	1730.6
HLA-DR2b $\beta$ -chain						
						ESI-MS*
						HALD-MS

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TABLE 5  
WT-20/HLA-DR3 NATURALLY PROCESSED PEPTIDES

Protein Source	Position	Sequence	Length	Fraction	MW	Mass Spec.
Pseudo HLA-A2 Apolipoprotein B-100 (Human)	103-117	VGSDVRLRGTHQYA	15	DR3-2-63	1855.0	1863.9
	1276-1295	HFLKSDGRIKYLKNSLK	20	DR3-2-63	2352.9	2360.0
	1273-1291	IPQHLFLKSDGRIKYLK	19	DR3-2-63	2235.5	2245.1
	1276-1291	HFLKSDGRIKYLK	16	DR3-2-60	1910.2	1911.4
	1276-1290	HFLKSDGRIKYLK	15	DR3-2-60	1782.1	1785.9
HLA-DR $\beta$ -chain Invariant chain (II)	1207-1224	YANILLDRRVPQDHTF	17	DR3-2-63	2053.3	2059.1
	1-18	GGTRPRFLEYSTSECHFF	18	DR3-2-73	?	?
	96-118	LPKPKPVSKRMATPLLHQAIP	23	DR3-2-73	2545.2	2554.0
	97-118	PKPKPVSKRMATPLLHQAIP	22	DR3-2-73	2432.0	2441.4
	98-118	KPKPVSKRMATPLLHQAIP	21	DR3-2-73	2334.9	2345.3
						MALD-MS

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TABLE 6  
PRIEST/HLA-DR6 NATURALLY PROCESSED PEPTIDES

PROTEIN SOURCE	POSITION	SEQUENCE	LENGTH	FRACTION	MW	MASS SPEC
Ig K $\alpha$ Chain C region (Human)	188-208	KHKVYACEVTHHGLSSPVTKS	21	DR4-2-45	2299.6	2304.0
	188-207	KHKVYACEVTHHGLSSPVTK	20	DR4-2-47	2212.5	2213.0
	189-206	HKVYACEVTHHGLSSPVT	18	DR4-2-43	1955.5	1952.1
	188-204	KHKVYACEVTHHGLSSP	17	DR4-2-45	1883.1	1882.8
	187-203	EKKVYACEVTHHGLSS	17	DR4-2-45	1915.1	1922.5
	188-203	KHKVYACEVTHHGLSS	16	DR4-2-54	1787.0	1787.0
	189-204	HKVYACEVTHHGLSSP	16	DR4-2-47	1755.0	1767.8
	187-202	EKKVYACEVTHHGLS	16	DR4-2-43	1828.0	1822.8
	188-202	KHKVYACEVTHHGLS	15	DR4-2-51	1699.9	1708.3
	189-203	HKVYACEVTHHGLSS	15	DR4-2-45	1657.8	1667.0
	187-200	EKKVYACEVTHHG	14	DR4-2-51	1628.8	1632.6
	182-198	APSPLPETTENVVVLCG	17	DR4-2-43	1697.9	1700
	28-48	VDDTGVRFDSDAASQRMPEPR	21	DR4-2-56	2470.6	2472.9
	28-47	VDDTGVRFDSDAASQRMPEP	20	DR4-2-59	2314.5	2319.3
HLA-A2	28-46	VDDTGVRFDSDAASQRMPE	19	DR4-2-54	2217.2	2218.7
	30-48	DTGVRFDSDAASQRMPEPR	19	DR4-2-55	2256.4	2263.2
	31-49	TGVRFDSDAASQRMPEPRA	19	DR4-2-56	2212.4	2211.5
	28-44	VDDTGVRFDSDAASQRMPE	17	DR4-2-55	1957.0	1963.1
	31-47	TGVRFDSDAASQRMPEP	17	DR4-2-56	1985.1	1987.5
	31-45	TGVRFDSDAASQRM	15	DR4-2-54	1758.9	1761.0
	31-42	TGVRFDSDAAS	12	DR4-2-54	1343.4	1343.3

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Table 6, continued

PROTEIN SOURCE	POSITION	SEQUENCE	LENGTH	FRACTION	MW	MASS SPEC
HLA-C	28-50	VDDTQFVRFDSDAASPRGEPRAP	23	DR4-2-56	2533.7	2536.7
	31-52	TQFVRFDSDAASPRGEPRAPW	22	DR4-2-54	2489.7	2491.5
	28-48	VDDTQFVRFDSDAASPRGEPR	21	DR4-2-54	2365.5	2368.1
	28-47	VDDTQFVRFDSDAASPRGEP	20	DR4-2-56	2209.3	2211.5
	28-46	VDDTQFVRFDSDAASPRGE	19	DR4-2-56	2112.2	2113.9
	28-45	VDDTQFVRFDSDAASPRG	18	DR4-2-56	1983.1	1987.5
	31-48	TQFVRFDSDAASPRGEPR	18	DR4-2-52	2036.2	2041.5
	28-44	VDDTQFVRFDSDAASPR	17	DR4-2-55	1926.0	1931.7
	30-46	DTQFVRFDSDAASPRGE	17	DR4-2-52	1897.9	1901.6
	31-44	TQFVRFDSDAASPR	14	DR4-2-52	1596.7	1603.7
	31-42	TQFVRFDSDAAS	12	DR4-2-54	1343.4	1343.3
HLA-C	130-150	LRSTAAADTAAGITQRKVEAA	21	DR4-2-56	2376.6	2376.4
	129-145	DLRSUTAAADTAAGITQR	17	DR4-2-59	1904.5	1908.7
	129-144	DLRSUTAAADTAAGITQ	16	DR4-2-59	1747.9	1752.3
	129-143	DLRSUTAAADTAAGIT	15	DR4-2-59	1619.7	1622.2
HLA-B*62	129-145	DLSSUTAAADTAAGITQR	17	DR4-2-60	1834.9	1838.1
	129-148	DLSSUTAAADTAAGITQRKVE	20	DR4-2-66	2278.4	2284.6
Cathepsin C (Rat Homologue)	151-167	YDHNFKVKAINDOKSVT I	17	DR4-2-70	2037.2	2039.6
	151-166	YDHNFKVKAINDOKSW I	16	DR4-2-70	2035.3	1937.7
HLA-DR $\beta$ -chain	1-14	GDTRPRFLEQVKHE	14	DR4-2-72	1936.1	
					1934.2	
IG Heavy Chain	121-7	GVYFYLGGRSTLVSVS (7)	(7)	DR4-2-6	7	7
MALD-MS						

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TABLE 7  
NAIIN/HLA-DR7 NATURALLY PROCESSED PEPTIDES

PROTEIN SOURCE	POSITION	SEQUENCE	LENGTH	FRACTION	MW	MASS SPEC
Pseudo HLA-A2	103-120	VGSDARFLRGVHQYADG	18	DR7-2-63	2190.4	2194
	103-117	VGSDARFLRGVHQYA	15	DR7-2-63	1855.0	1860
HLA-A29	234-253	RPAGDGTFOKIASVVPSCQ	20	DR7-2-66	2087.3	2092
	234-249	RPAGDGTFOKIASVVV	16	DR7-2-63	1717	1718
	237-258	GDGTFOKIASVVPSCGEQRTT	22	DR7-2-66	2436	2440
	237-254	GDGTFOKIASVVPSCQE	18	DR7-2-66	1892.3	1892
	239-252	GTFOKIASVVPSCG	14	DR7-2-66	1462	1465
	239-253	GTFOKIASVVPSCQ	15	DR7-2-66	1718	1721
	239-261	GTFOKIASVVPSCGEQRTCHV	23	DR7-2-66	2603	2606
	58-78	GALANIADVCKANLEINTKRSN	21	DR7-2-66	2229.5	2221
	38-54	TPSYVAFDTERLIGDA	17	DR7-2-69	1856.0	1856.6
	38-52	TPSYVAFDTERLIG	15	DR7-2-72	1656.0	1657.0
Heat shock cognate 71 kD protein	97-118	PKPKPVSKRMATPLLMAALP	22	DR7-2-72	2432.0	2436.6
	98-118	KPKPKVSKRMATPLLMAALP	21	DR7-2-72	2334.9	2339.7
Invariant chain (11)						
						MAID-MS

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TABLE 8  
Z3.1/HLA-DRB NATURALLY PROCESSED PEPTIDES

PROTEIN SOURCE	POSITION	SEQUENCE	LENGTH	FRACTION	MW	MASS SPEC
Metalloproteinase inhibitor 1 Prec. (Human)	102-117	RSEEFIAAGKLODGLL	16	DRB-3-70	1789.0	1799.9
	101-117	SEEFIAAGKLODGLL	15	DRB-3-72	1632.9	1646.0
Transferrin (Bov?)	261-275	DVIWELLHAGNFQ	15	DRB-3-78	1808.0	1818.1
	38-53	EPFLYILGKSRVLEAG	16	DRB-3-78	1863.2	1868.4
Calcitonin receptor (Hum?)	189-203	TAFOYIDNKGIDSD	15	DRB-3-63	1699.8	1711.0
	189-202	TAFOYIDNKGIDS	14	DRB-3-63	1584.7	1595.0
						MALD-MS

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TABLE 9  
HOM2/HLA-DR1 NATURALLY PROCESSED PEPTIDES

PROTEIN SOURCE	POSITION	SEQUENCE	LENGTH	FRACTION	PI	MASS SPEC
Pseudo HLA-A2	103-117	VGSDVRFGRGYHYA	15	H2/DR1-1-64	1855.0	1854.4
	104-117	GSDVRFGRGYHYA	14	H2/DR1-1-63	1755.3	1755.2
Invariant Chain (11)	96-119	LPKPKPVSKHRNATPLLMOALPH	24	H2/DR1-1-77	2676.4	2675.9
	97-120	PKPKPKVSKHRNATPLLMOALPHG	24	H2/DR1-1-72	2620.2	2619.7
	96-118	LPKPKPVSKHRNATPLLMOALP	23	H2/DR1-1-73	2545.2	2544.5
	97-119	PKPKPKVSKHRNATPLLMOALPH	23	H2/DR1-1-75	2503.2	2502.3
	98-119	KPKPKVSKHRNATPLLMOALPH	22	H2/DR1-1-75	2466.1	2465.8
	97-118	PKPKPKVSKHRNATPLLMOALP	22	H2/DR1-1-72	2432.0	2431.7
	98-118	KPKPKVSKHRNATPLLMOALP	21	H2/DR1-1-72	2334.9	2334.2
						ESI-MS

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TABLE 10  
HUMAN SPLEEN DR4/DR11 NATURALLY PROCESSED PEPTIDES

PROTEIN SOURCE	POSITION	SEQUENCE	LENGTH	FRACTION	MW	MASS SPEC
HLA-DR $\alpha$ -chain	133-156	SETVLPREDHLFRKFHYLPFLPS	24	FFR.391-71	2976.4	2982.5
	136-156	VFLPREDHLFRKFHYLPFLPS	21	FFR.391-71	2659.1	2665.9
	136-155	VFLPREDHLFRKFHYLPFLP	20	FFR.391-71	2572.0	2579.6
	136-151	VFLPREDHLFRKFHYL	16	FFR.391-71	2117.5	2126.6
Calgranulin B	25-50	KLGNPOTLNGGEFKELVRKOLQFLK	26	FFR.391-71	3068.5	3073.0
	25-48	KLGNPOTLNGGEFKELVRKOLQNF	24	FFR.391-71	2827.2	2831.8
	25-38	KLGNPOTLNGGEFK	14	FFR.391-71	1583.8	1591.2
						MALD-MS

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CLAIMS

1. A purified preparation of a peptide comprising an amino acid sequence identical to that of a segment of a naturally-occurring human protein, said segment being  
5 of 10 to 30 residues in length, inclusive, wherein said peptide binds to a human major histocompatibility complex (MHC) class II allotype.

2. The preparation of claim 1, wherein said peptide binds to at least two distinct MHC class II  
10 allotypes.

3. The preparation of claim 1, wherein said human protein is HLA-A2, HLA-A29, HLA-Bw62, HLA-C, HLA-DR $\alpha$ , HLA-DR $\beta$ , invariant chain (Ii), Ig kappa chain C region, Ig heavy chain, Na<sup>+</sup>/K<sup>+</sup> ATPase, transferrin, transferrin  
15 receptor, calcitonin receptor, carboxypeptidase E, MET kinase-related transforming protein, guanylate-binding protein, mannose-binding protein, apolipoprotein B-100, cathepsin C, cathepsin S, metalloproteinase inhibitor 1 precursor, or heat shock cognate 71 kD protein.

20 4. The preparation of claim 1, wherein said human protein is an MHC class I or II molecule.

5. The preparation of claim 1, wherein said segment conforms to the following motif:

at a first reference position (I) at or within 12  
25 residues of the amino terminal residue of said segment, a positively charged residue or a large hydrophobic residue; and

at position I+5, a hydrogen bond donor residue.

6. The preparation of claim 5, wherein said motif  
30 comprises a hydrophobic residue at I+9.

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7. The preparation of claim 6, wherein said motif additionally comprises, at position I+1 or I-1, a hydrophobic residue.

8. The preparation of claim 1, wherein said  
5 segment comprises residues 29-40 or residues 106-115 of HLA-A2.

9. The preparation of claim 1, wherein said segment comprises residues 107-116 of Ii.

10. A therapeutic composition comprising  
10 (a) a peptide comprising an amino acid sequence identical to that of a segment of a naturally-occurring human protein, said segment being of 10 to 30 residues in length, wherein said peptide binds to a human major histocompatibility complex (MHC) class II allotype; and  
15 (b) a pharmaceutically acceptable carrier.

11. A liposome containing a peptide comprising an amino acid sequence identical to that of a segment of a naturally-occurring human protein, said segment being of 10 to 30 residues in length, wherein said peptide binds  
20 to a human major histocompatibility complex (MHC) class II allotype.

12. An immune-stimulating complex (ISCOM) comprising a peptide comprising an amino acid sequence identical to that of a segment of a naturally-occurring  
25 human protein, said segment being of 10 to 30 residues in length, wherein said peptide binds to a human major histocompatibility complex (MHC) class II allotype.

13. A method of inhibiting an immune response in a human patient, which method comprises contacting an

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antigen-presenting cell (APC) of a patient with the therapeutic composition of claim 10.

14. A method of inhibiting an immune response in a human patient, which method comprises contacting an APC  
5 of a patient with the liposome of claim 11.

15. A method of inhibiting an immune response in a human patient, which method comprises contacting an APC of a patient with the ISCOM of claim 12.

16. A nucleic acid encoding a polypeptide, said  
10 polypeptide comprising a first and a second amino acid sequence linked by a peptide bond, said first sequence being identical to that of a segment of a naturally-occurring human protein, which segment binds to a human MHC class II allotype and is of 10 to 30 residues in  
15 length; and said second sequence being a sequence which controls intracellular trafficking of a polypeptide to which it is attached ("trafficking sequence").

17. The nucleic acid of claim 16, wherein said trafficking sequence traffics said polypeptide to  
20 endoplasmic reticulum (ER), a lysosome, or an endosome.

18. The nucleic acid of claim 16, wherein said second sequence is substantially identical to the signal peptide of an MHC subunit.

19. The nucleic acid of claim 18, wherein said  
25 subunit is an MHC class II  $\alpha$  or  $\beta$  subunit.

20. The nucleic acid of claim 16, wherein said trafficking sequence is KDEL; KFERQ; QREFK; MAISGVPVLGFFIIAVLMSAQESWA; a pentapeptide comprising Q

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flanked on one side by four residues selected from K, R, D, E, F, I, V, and L; or a signal peptide.

21. A liposome or ISCOM comprising the nucleic acid of claim 16.

5           22. A nucleic acid encoding a polypeptide comprising a first and a second amino acid sequence linked by a peptide bond, said first sequence being identical to that of a segment of a naturally-occurring human protein, which segment binds to a human MHC class  
10 II allotype and is of 10 to 30 residues in length; and said second sequence being substantially identical to human Ii.

23. The nucleic acid of claim 22, wherein said polypeptide comprises a plurality of copies of said first  
15 sequence linked in tandem to said second sequence.

24. A nucleic acid molecule encoding a self peptide comprising an amino acid sequence identical to that of a segment of a naturally-occurring human protein, said segment being of 10 to 30 residues in length,  
20 wherein said self peptide binds to a human major histocompatibility complex (MHC) class II allotype, and wherein said nucleic acid molecule encodes less than the entire sequence of said protein.

25. The nucleic acid molecule of claim 24,  
25 wherein said molecule additionally encodes a peptide sequence which controls intracellular trafficking of a polypeptide to which it is attached ("trafficking sequence").

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26. The nucleic acid molecule of claim 25,  
wherein said molecule additionally encodes a second self  
peptide and a second trafficking sequence.

27. The nucleic acid molecule of claim 24,  
5 wherein said molecule additionally comprises expression  
control elements.

28. The nucleic acid molecule of claim 24,  
wherein said molecule comprises plasmid or viral genomic  
sequence.

10 29. The nucleic acid molecule of claim 28,  
wherein said molecule is the genome of a non-replicative,  
non-virulent vaccinia virus, adenovirus, Epstein-Barr  
virus, or retrovirus.

30. A liposome or ISCOM comprising the nucleic  
15 acid molecule of claim 24.

31. A cell comprising the nucleic acid molecule  
of claim 27.

32. The cell of claim 31, wherein said cell is a  
human B cell or APC.

20 33. The cell of claim 31, wherein said nucleic  
acid comprises genomic nucleic acid of a virus.

34. A method of making a peptide, which method  
comprises culturing the cell of claim 31 under conditions  
permitting expression of said peptide from said nucleic  
25 acid molecule.

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35. A method of inhibiting an immune response in a human patient, which method comprises introducing the nucleic acid of claim 24 into a plurality of APCs of said patient.

5           36. A therapeutic composition comprising the nucleic acid of claim 24 in a pharmaceutically acceptable carrier.

37. A method of inducing an immune response in a human patient, which method comprises introducing into an  
10 APC of said patient a nucleic acid molecule encoding an immunogenic fragment of a protein of other than human origin, wherein said fragment binds to an MHC class I or II molecule.

38. The method of claim 37, wherein said protein  
15 is of an infective agent which causes human or animal disease.

39. The method of claim 38, wherein said infective agent is human immunodeficiency virus (HIV), hepatitis B virus, measles virus, rubella virus,  
20 influenza virus, rabies virus, *Corynebacterium diphtheriae*, *Bordetella pertussis*, *Plasmodium spp.*, *Schistosoma spp.*, *Leishmania spp.*, *Trypanasoma spp.*, or *Mycobacterium lepre*.

40. The preparation of claim 1, wherein said  
25 segment consists essentially of a sequence set forth in any of Tables 1-10.

41. A method of identifying a nonallelically restricted immunomodulating peptide, said method comprising:

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(a) fractionating a mixture of peptides eluted from a first MHC class II allotype;

(b) identifying a self peptide from said mixture;

(c) testing whether said self peptide binds to a  
5 second MHC class II allotype, said binding being an indication that said self peptide is a nonallelically restricted immunomodulating peptide.

42. A method of identifying a potential immunomodulating peptide, said method comprising:

10 (a) providing a cell expressing MHC class II molecules on its surface;

(b) introducing into said cell a nucleic acid encoding a candidate peptide;

(c) determining whether the proportion of said  
15 class II molecules which are bound to said candidate peptide is increased in the presence of said nucleic acid compared to the proportion bound in the absence of said nucleic acid, said increase being an indication that said candidate peptide is a potential immunomodulating  
20 peptide.

43. A method of identifying a potential immunomodulating peptide, said method comprising:

(a) providing a cell expressing MHC class II molecules on its surface;

25 (b) introducing into said cell a nucleic acid encoding a candidate peptide;

(c) determining whether the level of MHC class II molecules on the surface of said cell is decreased in the presence of said nucleic acid compared to the level of  
30 said molecules in the absence of said nucleic acid, said decrease being an indication that said candidate peptide is a potential immunomodulating peptide.

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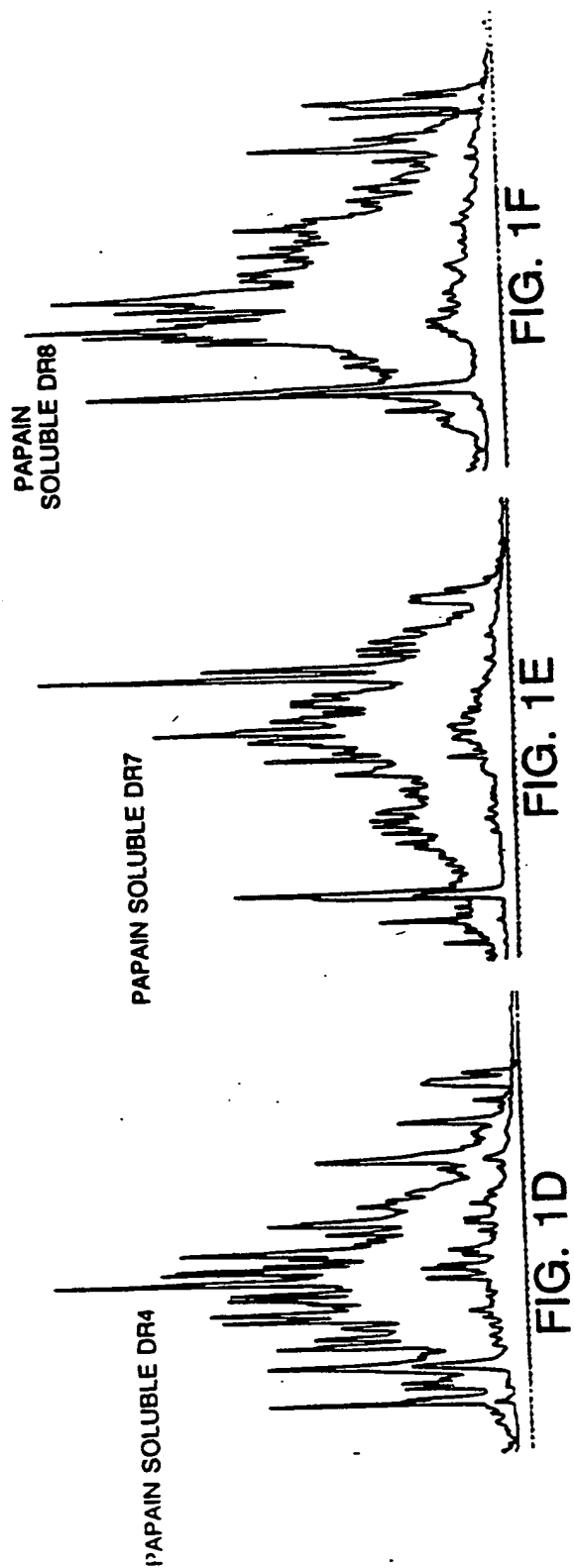
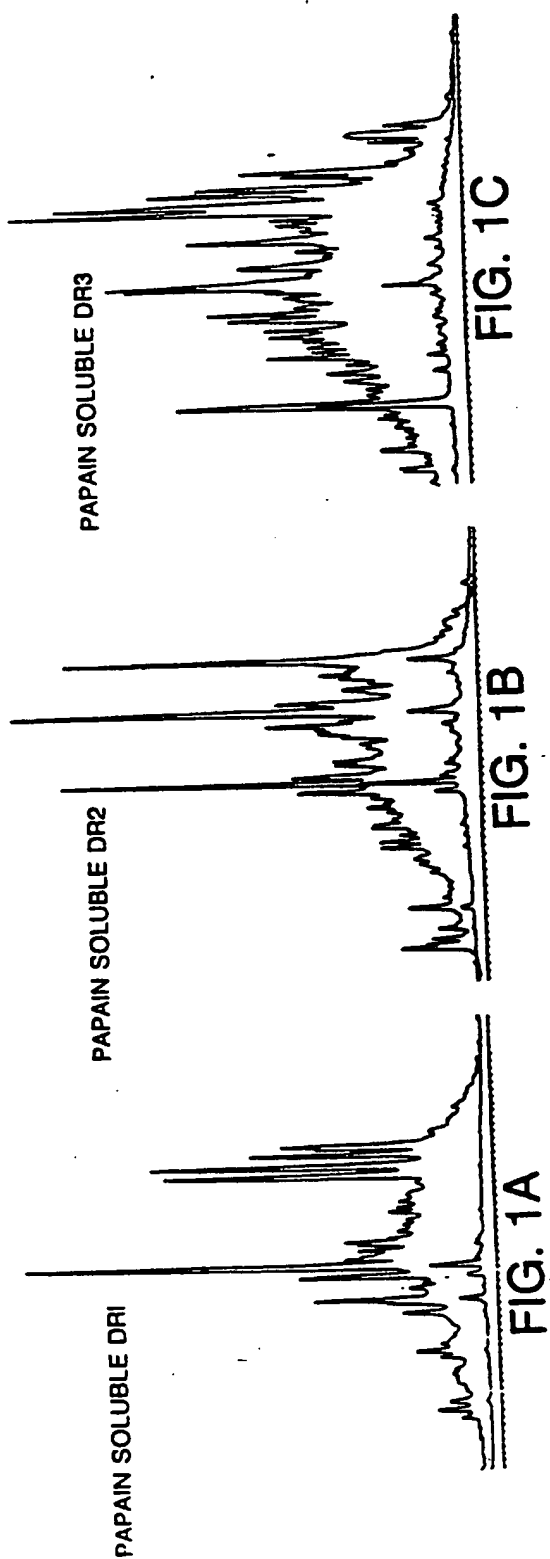
44. A method of identifying a nonallelically restricted immunostimulating peptide, said method comprising:

- (a) providing a cell bearing a first MHC class I  
5 or class II allotype, said cell being infected with a pathogen;
- (b) eluting a mixture of peptides bound to said cell's first MHC allotype;
- (c) identifying a candidate peptide from said  
10 mixture, said candidate peptide being a fragment of a protein from said pathogen;
- (d) testing whether said candidate peptide binds to a second MHC allotype, said binding being an indication that said candidate peptide is a  
15 nonallelically restricted immunostimulating peptide.

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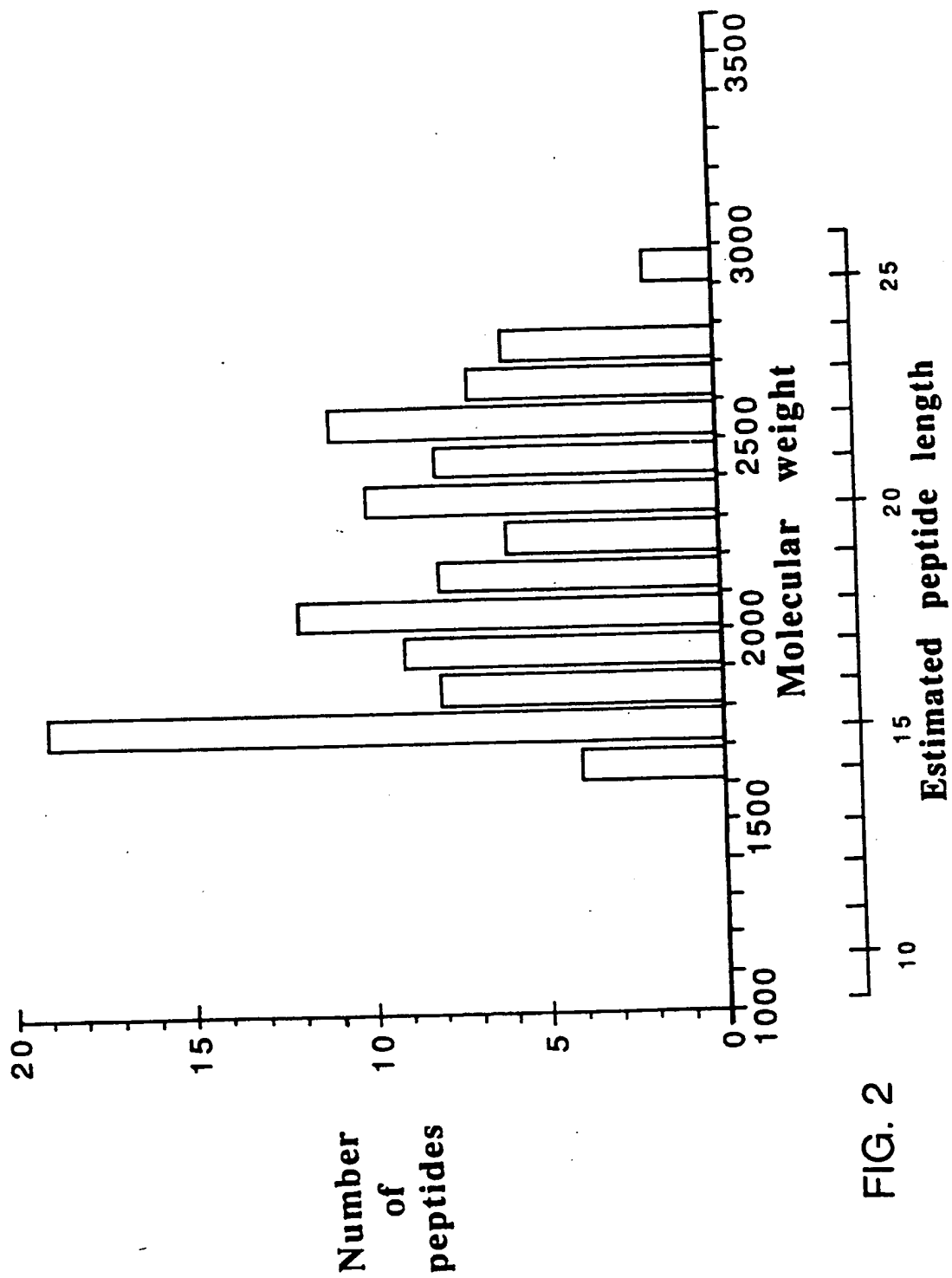


FIG. 2

3/3

ATG GCC ATA AGT GGA GTC CCT GTG CTA GGA TTT TTC ATC ATA GCT  
 M A I S G V P V L G F F I I A  
 GTG CTG ATG AGC GCT CAG GAA TCA TGG GCT AAG ATG CGC ATG GCC  
 V L M S A Q E S W A K M R M A  
 ACC CCG CTG CTG ATG CAG GCG CTG CCC ATG TAA  
T P L L M Q A L P M stop

FIG. 3A

ATG GCC ATA AGT GGA GTC CCT GTG CTA GGA TTT TTC ATC ATA GCT  
 M A I S G V P V L G F F I I A  
 GTG CTG ATG AGC GCT CAG GAA TCA TGG GCT CTT CCC AAG CCT CCC  
 V L M S A Q E S W A L P K P P  
 AAG CCT GTG AGC AAG ATG CGC ATG GCC ACC CCG CTG CTG ATG CAG  
K P V S K M R M A T P L L M Q  
 GCG CTG CCC ATG TAA  
A L P M stop

FIG. 3B

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/06692

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC(5) : A61K 39/00, 39/385, 37/22; C07K 7/00; C12N 5/12 US CL : Please See Extra Sheet. According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) U.S. : 424/88, 450; 530/300; 536/27 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched none Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) none		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US, A, 5,130,297 (Sharma, et al.) 14 July 1992. See entire document.	<u>1,2,10,11-15</u> 1-44
A	US, A, 4,681,760 (Fathman) 21 July 1987, see entire document.	1-44
A	Critical reviews in Immunology, vol. 11(5), issued 1992, J.C. Gorge, "Structural analysis of class II major histocompatibility complex proteins," pages 305-335, see entire document.	1-44
A	US, A, 4,478,823 (Sanderson) 23 October 1984. See entire document.	1-44
A	Immunol. Res., vol. 6, issued 1987, D.H. Margulies et al. "Engineering soluble major histocompatibility molecules: why and how", pages 101-116, see entire article.	1-44
A	NATURE, vol. 348, issued 13 December 1990, F.M. Brodsky, "The invariant chain service", pages 581-582.	1-44
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be part of particular relevance "B" earlier document published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reasons (as specified) "O" documents referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "A" document member of the same patent family		
Date of the actual completion of the international search 18 SEPTEMBER 1992		Date of mailing of the international search report 30 SEP 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE		Authorized officer THOMAS M. CUNNINGHAM Telephone No. (703) 308-0196